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ORIGINAL ARTICLE

Development of the species-specific multiplex PCR and DNA sequencing methods for rapid authentication of Isatidis Folium and its adulterants

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Abstract

***Isatis indigotica* Fort. (family Cruciferae), is an herb widely used in traditional herbal medicine and its dried leave was named "ISATIDIS FOLIUM". *Baphicacanthus cusia* (Ness) Bremek. and *Polygonum tinctorium* Ait. are commonly misused as ISATIDIS FOLIUM in Chinese Medicine pharmacy. For the purpose of being not misused, specific primers based on the sequence difference of chloroplast *trnH-psbA* intergenic spacer were designed and multiplex polymerase chain reaction method (multiplex PCR) was developed. In this study, 29 original herbal materials were analyzed and our results show that DNA size after multiplex PCR was able to distinguish variations between three herbs. DNA fragments of 464, 297, 170 base pairs (bps) were represented for *I. indigotica* and *B. cusia* and *P. tinctorium*, respectively. In conclusion, our investigations demonstrate that molecular identification method provides more accurate results for medicinal plants detection and good quality control of ISATIDIS FOLIUM.**

Keywords: DNA sequencing, ISATIDIS FOLIUM, Multiplex PCR, *TrnH-psbA* intergenic spacer

1. Introduction

According to the third edition of the Taiwanese Pharmacopoeia, ISATIDIS FOLIUM is the dried leaf of the Cruciferae plant *Isatis indigotica* Fort. [1], namely "Daqingye". It is a common traditional herbal medicine with the effect of detoxification. In addition, several studies had revealed several pharmacologic functions including anti-bacterial [2], anti-inflammatory [3] and anti-viral [4–6] activities. However, "Daqingye" is an easily confused herb and its adulteration often occurs in markets. Besides, at least three different medicinal plants are also referred to "Daqingye" including *I. indigotica* Fort., (Cruciferae), *Baphicacanthus cusia* (Nees) Bremek. (Acanthaceae), and *Polygonum tinctorium* Ait. (Polygonaceae) in Chinese Medicine pharmacy. The same natural dye is extracted from these three plant species, which may explain why these herbal plants shared the common name [7,8]. The botanical origin of "Daqingye" was different plus the divergence of regional usage leading to the misuse situation of alternate. These misuse situations may affect the pharmacological efficacy and medicinal safety, which must be avoided. Therefore, an accurate and convenient identification method/tool to distinguish the authentic medicinal ISATIDIS FOLIUM from its adulterants is absolutely essential.

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To ensure the quality of medicinal effects of Chinese herbal medicines, the first step is to choose the correct medicinal materials; however these raw materials must be scientifically identified. Due to the more rigorous identification of original phytochemicals and active ingredients are done, the more safety of the subsequent products can be accepted. In recent years, DNA-based molecular markers have been widely used as powerful tools for herbal plants identification [9,10] due to unique genetic characteristics and have more advantages than other marker systems of being not affected by age, environmental factors and physiological conditions. Only a small amount of sample is sufficient for analysis and the physical form of the sample does not restrict detection [11,12]. To assure the correct herb material authentication, the multiplex PCR comprises multiple primer sets within a single PCR reaction to specific amplify DNA fragments of each target template. Amplified PCR product could be separated by the gel electrophoresis and purified for DNA sequencing, which can be applied to distinguish medicinal plant species and evolutionary relationships. These molecular techniques have been extensively used as accurate, reproducible and reliable tools to identify medicinal plant species [13–17]. The DNA-based authentication provides a reliable method to identify species adulteration, especially for Traditional Chinese Medicine (TCM). The purpose of this study was to explore effective and quick approaches to discriminate the genuine origin of ISATIDIS FOLIUM and its adulterants for ensuring the quality of raw materials in the market.

2. Materials and methods

2.1. Materials

Three specimens of *I. indigotica* (j-1-2-07), *B. cusia* (L-3-3-02) and *P. tinctorium* (j-2-1-01), deposited at the herbarium of the Food and Drug Administration, Ministry of Health and Welfare, Taipei, Taiwan, R.O.C., had been confirmed by our researchers and used as the standard raw materials. 29 samples were dried leaves and non-processed. These samples were collected from commercial suppliers in Taiwan and China and twenty-three of which were the vernacular name "Daqingye", four were *B. cusia*, and two were *P. tinctorium*.

2.2. Genomic DNA extraction

The genomic DNA was extracted according to the ionic detergent cetyltrimethylammonium bromide (CTAB) methods with some modifications [18,19]. Prepare 100 mg of the leaf powder and transferred into a 2 mL sterile centrifuge tube. Add 1 mL lysis buffer (100 mM Tris–HCl, 100 mM EDTA, 1% sodium lauroylsarcosinate, pH 8.0) containing proteinase K (1 mg/mL) into tube and incubate at 56 °C for 1 h. After incubation, the solution was mixed with 1 mL of phenol/chloroform/isoamyl alcohol (25: 24: 1, v/v/v) and centrifuged at 12,000×*g* for 5 min (min). Transfer 0.7 mL upper aqueous phase carefully into new tube and add 0.14 mL of 5 M NaCl and 0.1 mL of 10% CTAB in 0.7 M NaCl and incubated at 65 °C for 15 min. After incubation, add 1 mL chloroform/isoamyl alcohol (24: 1, v/v) and centrifuged at 12,000×*g* for 5 min. Transfer upper aqueous phase into new tube and add 0.1 volume sodium acetate solution (3M, pH5.5) and 0.7 volume of isopropanol (−20 °C), followed by gentle inversion and centrifuged at 12,000×*g* for 5 min. After centrifugation, carefully decant the supernatant and air-dry DNA pellet as well as dissolve pellet with 0.1 mL of sterile distilled water. In addition, GFX™ DNA and Gel Band Purification Kit (GE healthcare, Pittsburgh, PA, USA) were used to further purify dissolved DNA. DNA quality and concentration were measured by NanoDrop spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) and Qubit 4.0 fluorometer (Thermo Scientific, Wilmington, Delaware, USA) for the further polymerase chain reaction (PCR) and sequencing.

2.3. Primers design

The chloroplast *trnH-psbA* intergenic spacer sequences of three target species were obtained from the GenBank database as shown in Fig. 1. Accession numbers were as following: *I. indigotica* (GQ435333); *B. cusia* (KT161373); *P. tinctorium* (EU554055). We also amplified and sequenced the *trnH-psbA* intergenic spacer region of standard herbal materials (j-1-2-07, L-3-3-02 and j-2-1-01) with the universal primers PA and TH [20]. These sequences of the standard herbal materials in our study (j-1-2-07, L-3-3-02 and j-2-1-01) were verified with other herbal species on GenBank database using the BLAST search tool. All sequences were aligned using MAFFT version 7.402 software [21] and species-specific primers were designed manually based on the difference of the sequences (Fig. 1).

2.4. PCR condition

Each primer set (listed in Table 1) was analyzed with PCR under the same amplification condition. To test the primer specificity, the mixing powders

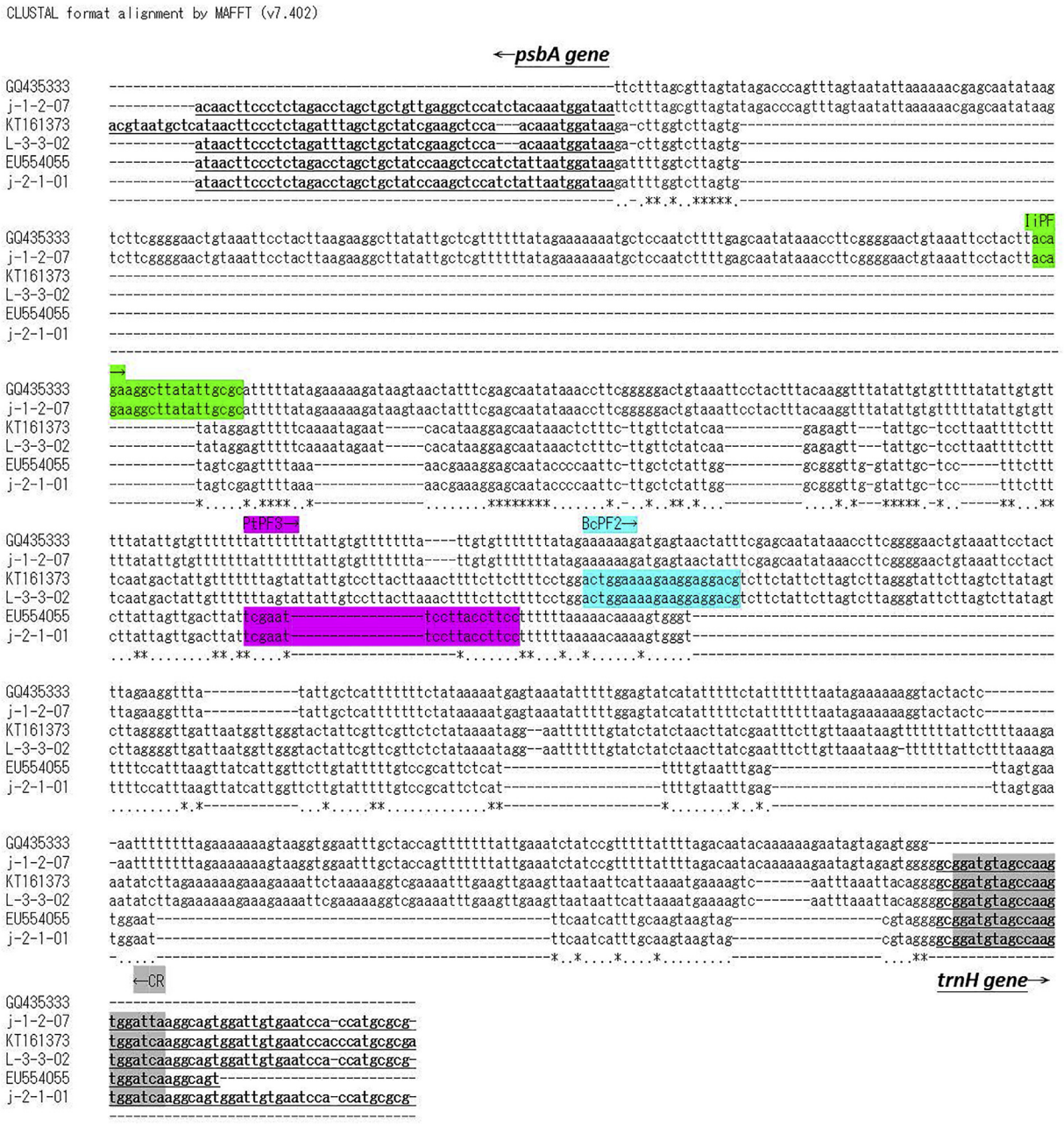

Fig. 1. Sequence alignment of trnH-psbA intergenic spacer region. Different regions of trnH-psbA intergenic spacer sequences between Isatis indigotica, Baphicacanthus cusia and Polygonum tinctorium were used to design species-specific primers. NCBI GenBank Accession numbers: GW435333 (I. indigotica), KT161373 (B. cusia), EU554055 (P. tinctorium). Standard raw materials number: j-1-2-07 (I. indigotica), L-3-3-02 (B. cusia), j-2-1-01 (P. tinctorium). Every letter element in the sequence alignment analysis is either a match or nonmatch or a gap. The dash symbol represented "gap" in alignment sequences and the asterisk symbol represented "match" in alignment sequences. The dot symbol represented "nonmatch" in alignment sequences.

Table 1. Primer information of multiplex PCR.

Primer Direction	Primer Name	Primer Sequence (5′ → 3′)	Specificity (target Species)	PCR product size (base pairs)
Forward	IiPF	ACAGAAGGCTTATATTGCGC	*Isatis indigotica*	464
	BcPF2	ACTGGAAAAGAAGGAGGACG	*Baphicacanthus cusia*	297
	PtPF3	TCGAMTTCCTKACCTTCC	*Polygonum tinctorium*	170
Reverse	CR	TRATCCACTTGGCTACATCC	All three target species	

comprise *I. indigotica*, *B. cusia* and *P. tinctorium* were prepared with the same ratio as well to imitate possibility of com-material adulteration and substitution. Amplification was performed using Astec PC320 thermal cycler (Astec, Fukuoka, Japan) of total 50 μL reaction mixture solution containing 2 μL genomic DNA, 10 μL Fast-Run™ Taq Master Mix (Protech Technology, Taipei, Taiwan), 0.5 μL of 25 μM PCR primers and 37 μL sterilized distilled water. The reaction conditions were as follows: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s (sec), 50 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. This experiment was verified with a negative control (sterile ultra-pure water) and a positive control (The DNA mixture of *I. indigotica*, *B. cusia* and *P. tinctorium*). The PCR reaction employed universal primers for the high copy number nuclear gene (*18S ribosomal ribonucleic acid*, *18S rRNA*) to provide the internal positive control for DNA extraction. We amplified the *18S rRNA* with universal primers [22] under the conditions following: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min.

2.5. Multiplex PCR assay

Multiplex PCR amplification was performed using Astec PC320 thermal cycler (Astec, Fukuoka, Japan) in 50 μL of reaction mixture solution containing 2 μL genomic DNA with 10 μL Fast-Run™ Taq Master Mix (Protech Technology, Taipei, Taiwan), 0.16, 0.16, 0.32 and 0.36 μL of 25 μM PCR primer (IiPF, BcPF2, PtPF3 and CR), and sterilized distilled water. This experiment was verified using with a negative control (sterile ultra-pure water) and a positive control (The DNA mixture of *I. indigotica*, *B. cusia* and *P. tinctorium*). The reaction conditions were as follows: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. Samples for sensitivity assays are the mixing powders comprise *I. indigotica*, *B. cusia* and *P. tinctorium* were prepared with the same ratio as well to imitate possibility of com-material adulteration and substitution. Serial dilutions of DNA mixture (50 ng, 20 ng, 10 ng, 1 ng, 0.1 ng and 0.01 ng per microliter) of three target species were added in the respective reaction.

2.6. Electrophoresis

PCR products were analyzed using 2% agarose gel electrophoresis, stained with EZ-Vision™ Two Dye as Loading Buffer (AMRESCO, Solon, OH, USA) with the inclusion of a EZ-Vision 100 base pairs (bp) DNA ladder (AMRESCO, Solon, OH, USA) and visualized under ultraviolet (UV) illumination.

2.7. Sequence analysis

For sequencing analysis, 5 μL PCR products were incubated with 2 μL of ExoSAP-IT reagent (Affymetrix, Cleveland, OH, USA) at 37 °C for 15 min to remove excess dNTP and residual primers. Afterwards, the mixture was heated at 80 °C for 15 min to inactive the enzymes and bi-directional sequencing were conducted with BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, Carlsbad, California, USA) as well as ABI 3130 Genetic Analyzer (Applied Biosystems, Foster, CA, USA).

2.8. Species identification

Sequencing results were assembled using in MEGA version 6.0 and sequence analysis and

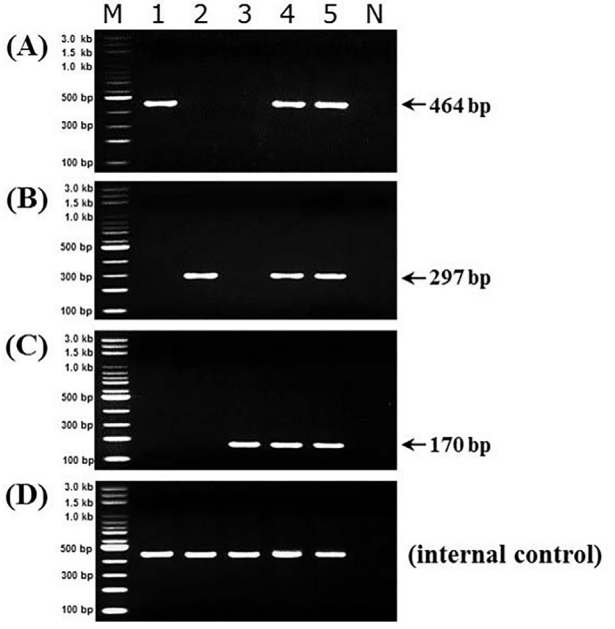

Fig. 2. The specificity assay of the species-specific primers in multi-single PCR reaction. (A) The specificity of IiPF-CR; (B) The specificity of BcPF2-CR; (C) The specificity of PtPF3-CR; (D) 18S rRNA internal control; Lane M, 100 bp DNA ladder marker; Lane 1, DNA of Isatis indigotica standard raw material (j-1-2-07); Lane 2, DNA of Baphicacanthus cusia standard raw material (L-3-3-02); Lane 3, DNA of Polygonum tinctorium standard raw material (j-2-1-01); Lane 4, DNA of the powder mixtures of I. indigotica, B. cusia and P. tinctorium (50 mg + 50 mg + 50 mg); Lane 5, DNA mixtures (50 ng/μL) by mixing the templates of I. indigotica (j-1-2-07), B. cusia (L-3-3-02) and P. tinctorium (j-2-1-01); Lane N, negative control.

species identification were performed by the BLAST search in the GenBank database.

3. Results

Species-specific primers designed manually from variable region are shown in Fig. 1. Three of forward species-specific primers designed from sequence-variable regions were paired with a single reverse primer (Table 1). We first evaluate the specificity of the primer pairs with DNA fragments amplified from three target species. According to electrophoresis results (Fig. 2) and sequencing data (Fig. 3), species-specific primers generated (amplified) specific DNA fragments of 464, 297 and 170 base pairs for *I. indigotica*, *B. cusia*, and *P. tinctorium*, respectively and no primer dimers or nonspecific amplicons were observed. The amplified PCR products were sequenced at ABI 3130 Genetic Analyzer. These nucleotide sequences were analyzed on the GenBank database using BLAST search tool for species identification. The specific DNA fragment of 464 bps showed 95% coverage and 100% similarity to the *I. indigotica* (Accession No. GQ435333). The specific DNA fragment of 297 bps showed 100% coverage and 99% similarity to the *B. cusia* (Accession No. KT161373). The specific DNA fragment of 170 bps showed 100% coverage and 100% similarity to the *P. tinctorium* (Accession No. EU554055). Thus, we validated the rigorous specificity of primers and eventually developed a multiplex PCR assay.

In order to assess the sensitivity of the multiplex PCR, the detection limit of the multiplex PCR was performed with different dilution of template. We first testify the performance/expression of the multiplex PCR method under serial dilution of each DNA template (50 ng, 20 ng, 10 ng, 1 ng, 0.1 ng and 0.01 ng per microliter) of three target species and found that all three amplicons were detectable even

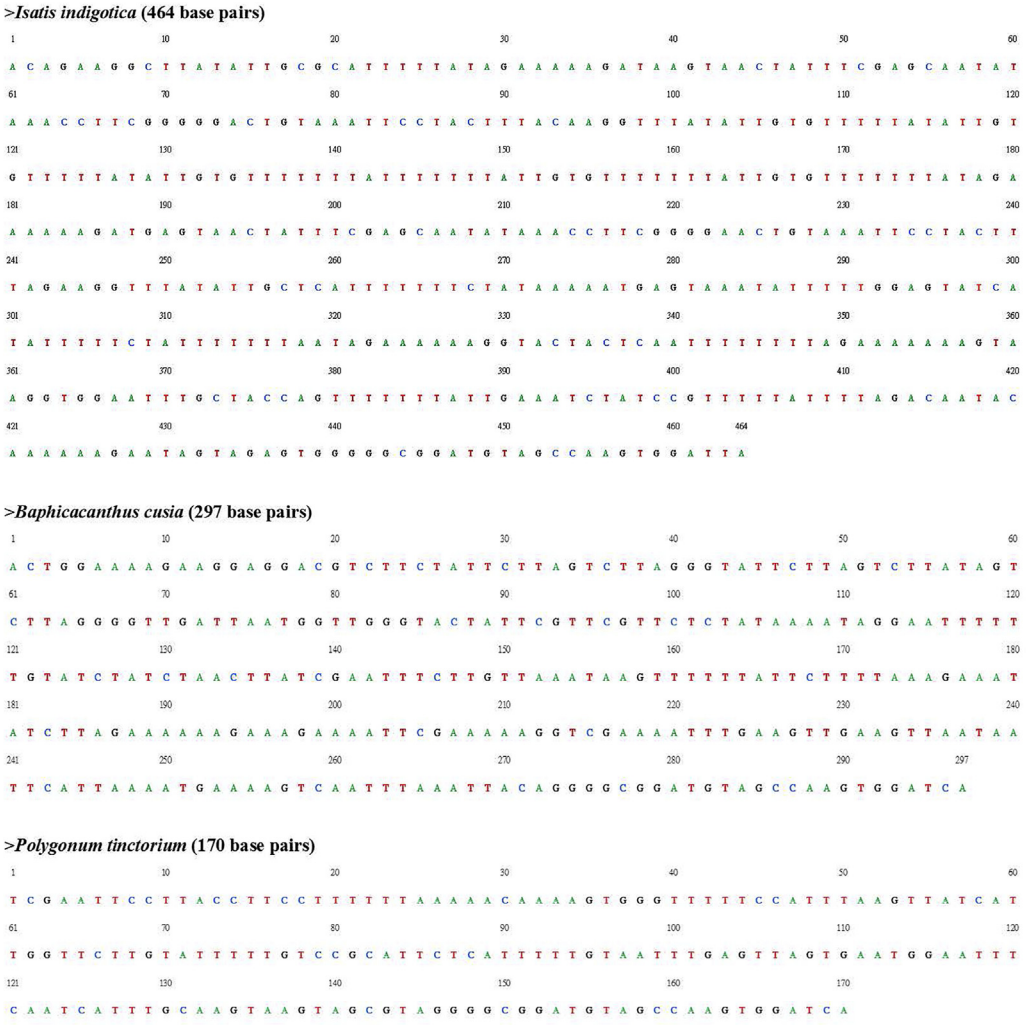

Fig. 3. Nucleotide sequences of I. indigotica, B. cusia, and P. tinctorium multiplex PCR-DNA sequencing data.

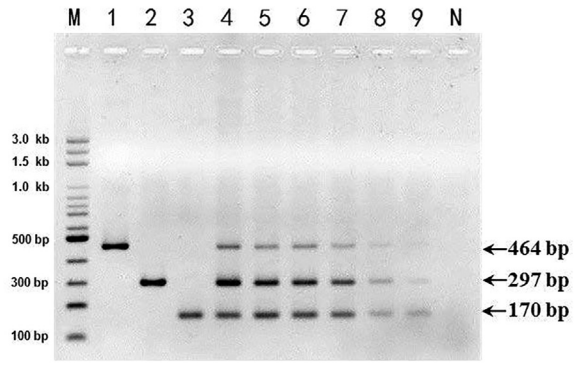

Fig. 4. DNA gel electrophoresis of multiplex PCR. DNA gel analysis showing the effectiveness of the multiplex PCR of individual target species (controls) with serial dilutions DNA mixtures (50 ng, 20 ng, 10 ng, 1 ng, 0.1 ng and 0.01 ng per microliter) by mixing the templates of Isatis indigotica (j-1-2-07), Baphicacanthus cusia (L-3-3-02) and Polygonum tinctorium (j-2-1-01). Lane M, 100 bp DNA ladder marker; Lane 1, DNA of I. indigotica standard raw material (j-1-2-07); Lane 2, DNA of B. cusia standard raw material (L-3-3-02); Lane 3, DNA of P. tinctorium standard raw material (j-2-1-01); Lane 4, DNA mixtures (50 ng/μL); Lane 5, DNA mixtures (20 ng/μL); Lane 6, DNA mixtures (10 ng/μL); Lane 7, DNA mixtures (1 ng/μL); Lane 8, DNA mixtures (0.1 ng/μL); Lane 9, DNA mixtures (0.01 ng/μL); Lane N, negative control.

as DNA template amounts was as low as 0.1 ng/μL (Fig. 4). This assay was proved to be highly sensitive for amplification of low quantities of DNA and thus could be applied to Isatidis Folium adulterants.

The multiplex PCR-DNA sequencing method was used to test 29 commercial raw material samples, and results were shown in Table 2. Among the 23 samples labeled "Daqingye", 11 samples were identified as *I. indigotica*, 11 samples as *B. cusia*, and 1 sample as *P. tinctorium*. These results demonstrated that multiplex PCR-DNA sequencing methods developed in this study will be useful for rapid and effective detection of adulterated medicines such as authentic ISATIDIS FOLIUM and its adulterants.

4. Discussion

The misuse of medicinal materials affects the quality of herbal medicine and also causes inconsistent therapeutic effects. Quality control has always been the key issue in the development and authentication of herbal medicines traded as bulk product. So far, many developed countries have

Table 2. Identification of raw material samples of "Daqingye" herb, Baphicacanthus cusia and Polygonum tinctorium by multiplex PCR-DNA sequencing method.

Raw material sample name	Sample number	Locality	Identification results of the multiplex PCR-DNA sequencing methods
"Daqingye" herb	IF01	Taipei, Taiwan	*Baphicacanthus cusia*
	IF02	Taipei, Taiwan	*Baphicacanthus cusia*
	IF03	Taipei, Taiwan	*Baphicacanthus cusia*
	IF04	Taipei, Taiwan	*Baphicacanthus cusia*
	IF05	Taipei, Taiwan	*Baphicacanthus cusia*
	IF06	Taipei, Taiwan	*Baphicacanthus cusia*
	IF07	Taipei, Taiwan	*Baphicacanthus cusia*
	IF08	Beijing, China	*Polygonum tinctorium*
	IF09	Beijing, China	*Isatis indigotica*
	IF10	Taipei, Taiwan	*Isatis indigotica*
	IF11	Taichung, Taiwan	*Isatis indigotica*
	IF12	New Taipei City, Taiwan	*Baphicacanthus cusia*
	IF13	Tainan, Taiwan	*Baphicacanthus cusia*
	IF14	Kaohsiung, Taiwan	*Baphicacanthus cusia*
	IF15	Kaohsiung, Taiwan	*Isatis indigotica*
	IF16	Tainan, Taiwan	*Isatis indigotica*
	IF17	Internet market, Taiwan	*Isatis indigotica*
	IF18	Tainan, Taiwan	*Isatis indigotica*
	IF19	Taipei, Taiwan	*Baphicacanthus cusia*
	IF20	Taoyuan, Taiwan	*Isatis indigotica*
	IF21	Hebei, China	*Isatis indigotica*
	IF22	Hebei, China	*Isatis indigotica*
	IF23	Taichung, Taiwan	*Isatis indigotica*
Baphicacanthus cusia	BC01	Taipei, Taiwan	*Baphicacanthus cusia*
	BC02	Taipei, Taiwan	*Baphicacanthus cusia*
	BC03	Taichung, Taiwan	*Baphicacanthus cusia*
	BC04	New Taipei City, Taiwan	*Baphicacanthus cusia*
Polygonum tinctorium	PT01	Beijing, China	*Polygonum tinctorium*
	PT02	Taichung, Taiwan	*Polygonum tinctorium*

been accepted the genomic fingerprinting analysis as a useful tool to qualify multicomponent herbal medicines and their final products. To increase the accuracy and practicality of fingerprinting analysis, proceeding to set up a comprehensive herbal plant genome database with standard herbal samples which were under taxonomical and official herbarium validation should be considered.

Identification of the original sources of medicinal plants is closely associated with their functional effects and medicinal safety. However, the commercial "Daqingye" materials have been dried or processed, thus making it difficult for consumers to immediately distinguish these herbs by their appearance. Previously study had demonstrated that three "Daqingye" plants can be distinguished by internal leaf anatomy analysis and chemical analysis of major constituents [23]. However, the microscopic examination of related plant species often possess similar features. Moreover, chemical varieties within the plant often hinder its botanical identification due to the chemical composition was affected by growth and storage conditions as well as by the harvesting process. However, DNA is an extremely stable macromolecule that is not affected by external factors and therefore can be recovered from fresh, dried and even processed biological materials. In addition, DNA markers are not tissue-specific and thus can be detected at any stage during organism development and only a small amount of sample is sufficient for analysis [24].

The species-specific multiplex PCR consists of multiple primer sets within a single PCR reaction, and by conducting the multiplex PCR, we can compare different sizes of DNA amplicons in a comprehensive manner at once according to different DNA templates. To have better results, annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction and DNA size for each target can be clearly discriminated after gel electrophoresis. In this study, we developed a multiplex PCR method to simultaneously identify *I. indigotica*, *B. cusia*, and *P. tinctorium* chloroplast *trnH-psbA* intergenic spacer sequences. Our results demonstrated this highly specific, sensitive, and rapid method can be applied for the simultaneous detection of ISATIDIS FOLIUM and its adulterants. In conclusion, we developed powerful molecular inspection methods for herbal materials, including multiplex PCR-DNA and sequencing, which are highly effective and stable, and could be applied for the quality control of the medicinal herb trades/bargains in markets.

Acknowledgements

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